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Inhibition of Gene Expression with Ribozymes

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SUMMARY

1. Ribozymes can be designed to cleave *in trans*, i.e. several substrate molecules can be turned over by one molecule of the catalytic RNA. Only small molecular weight ribozymes, or small ribozymes, are discussed in this review with particular emphasis on the hammerhead ribozyme as this has been most widely used for the inhibition of gene expression by cleavage of mRNAs.

2. Cellular delivery of the ribozyme is of crucial importance for the success of inhibition of gene expression by this methodology. Two modes of delivery can be envisaged, endogenous and exogenous delivery. Of the former several variants exist, depending on the vector used. The latter is still in its infancy, even though chemical modification has rendered such ribozymes resistant against degradation by serum nucleases without impairment of catalytic efficiency.

3. Various successful applications of ribozymes for the inhibition of gene expression are discussed, with particular emphasis on HIV1 and cancer targets. These examples demonstrate the promise of this methodology.

INTRODUCTION

Ribozymes are RNA molecules with the capacity to cleave a phosphodiester bond catalytically in a sequence-specific manner (Cech, 1990; Symons, 1992). The group I intron is used in pre-mRNA splicing, whereas the hammerhead, the hairpin, and the hepatitis delta ribozymes are utilized in the rolling circle replication of certain small pathogens of plants and animals. Natural cleavage occurs via an intramolecular or self-cleaving pathway, often termed *in cis*, whereby one strand of

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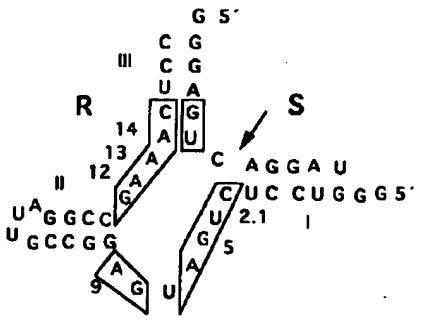

Fig. 1. Structure of a hammerhead ribozyme annealed to its substrate. R, ribozyme; S, substrate; arrow, position of cleavage. Numbering according to Hertel *et al.* (1992). Boxed nucleotides are invariant. I, II, and III denote helical regions.

RNA contains both the enzyme and the substrate portions of the complex. However, the reaction can be made intermolecular, or *in trans*, as shown in Fig. 1 for the hammerhead ribozyme (Hasselhoff and Gerlach, 1988; Uhlenbeck, 1987). In such constructs multiple turnover of the ribozyme can occur, i.e., one molecule of ribozyme can cleave more than one molecule of substrate, thus resembling protein enzymes. In addition to these ribozymes, there is the RNA component of RNaseP, which also acts catalytically and normally cleaves tRNA precursors (Yuan and Altman, 1994). This review concentrates on a discussion of the hammerhead ribozyme, as it is the most widely studied for the inhibition of gene expression. It has also been the subject of recent reviews (Bratty *et al.*, 1993; Long and Uhlenbeck, 1993).

For the inhibition of gene expression the cleaving complex is considered as being composed of the targeted mRNA, representing the substrate part, and the ribozyme, representing the enzyme part of the complex. The cleavage catalyzed by the hammerhead ribozyme occurs at a specific nucleotide triplet to generate a nucleoside 2′,3′-cyclic phosphate and a nucleoside with a free 5′-hydroxyl group. The hammerhead ribozyme cleaves on the 3′ side of a NUH triplet (N, any nucleotide; H,A,C,U) but, particularly efficiently, a GUC triplet (Hasseloff and Gerlach, 1988; Ruffner *et al.*, 1990; Perriman *et al.*, 1992; Nakamaye and Eckstein, 1994). On the other hand, the hairpin ribozyme cleaves at the 5′ side of a GUC triplet (Berzal *et al.*, 1993). Although only a limited number of triplets is susceptible to cleavage, it is highly likely that such sequences would occur several times in an RNA several hundred nucleotides in length, e.g., mRNA. Therefore most targets will contain a suitable triplet for cleaving. However, one prerequisite which must be met is that the cleavage triplet and the 5′ and 3′ recognition sequences must be easily accessible to the ribozyme and not otherwise involved in secondary structures, thus rendering it less accessible and potentially uncleavable.

Recognition of cleavable targets by ribozymes is achieved by complementary base pairing between the substrate and the arms of the ribozyme to form subsequently helices I and III (Fig. 1). From this complementary interaction the ribozyme could be thought of as adopting an RNA antisense approach but has the advantage of destroying the target once it is recognised.

This review discusses cellular delivery of the ribozyme and the associated problems of instability toward nucleases and the kinetic constraints. Finally, examples of successful inhibition of gene expression using ribozymes are

reviewed. Several recent reviews also cover the prospects of application of ribozymes to inhibit gene expression (Altman, 1993; Bratty *et al.*, 1993; Cotten, 1990; Rossi *et al.*, 1992; Rossi and Sarver, 1990). There are no reports up to now of the use of ribozymes in the area of neurobiology. However, antisense oligodeoxynucleotides have been successfully applied in this area (for recent publications see Kirch *et al.*, 1993; Osen-Sand *et al.*, 1993; Soreq *et al.*, 1994). It can thus be anticipated that the potential advantages of ribozymes will soon also be exploited in this field.

CELLULAR DELIVERY OF RIBOZYMES

The intracellular transcription of a ribozyme coding gene is termed endogenous ribozyme delivery. An alternative, exogenous delivery, is where the ribozymes are introduced to the cells either directly by injection, simply by addition to the cultured cells, or by transfection. This requires external preparation of the ribozyme either by chemical synthesis or by *in vitro* transcription. Problems of intracellular instability and translocation are common to both delivery methods, although different approaches are utilized to overcome these.

Endogenous Delivery

Endogenous delivery of ribozymes requires a vector containing the gene for the ribozyme behind a suitable promoter. The vector can be either a plasmid, a retroviral vector, or viral vectors such as that from adenovirus. Several reviews on gene therapy discuss the advantages and disadvantages of the various vectors used for the delivery of genes into cells (Miller, 1992; Morgan and Anderson, 1993; Mulligan, 1993).

Although these articles do not explicitly address the problem of delivery of ribozymes, many of the arguments also apply to these molecules. Transcription of the ribozyme can then be achieved from either a stably transfected or transduced cell line, i.e., where integration of the ribozyme gene into the genome has occurred, or from cells which have been transiently transfected with a plasmid carrying the ribozyme gene. It is imperative that the ribozyme gene is placed downstream of a strong promoter so that it is highly expressed in the cell. This ensures a high concentration of ribozyme so that substrate binding is not limiting.

The major advantages of the endogenous application of ribozymes lies in its continual expression. Depending upon the promoter used, the ribozyme will be transcribed only when the promoter is switched on (Zhao and Pick, 1993) (see Cancer and Other Targets, below). As the ribozyme is transcribed in the nucleus, it is expected to be transported into the cytoplasm like the mRNA to which it is targeted. Contrary to this expectation, pol III transcripts of a coupled ribozyme–tRNA gene, when injected into frog oocytes, remained mainly in the nucleus (Cotten and Birnstiel, 1989). If stable integration occurs, there is the additional possibility of generating transgenic organisms. However, integration of foreign DNA into the host genome could represent a dangerous intervention into the

genetic structure of the recipient, due to the possible activation of protooncogenes.

Exogen us Delivery

Exogenous delivery of ribozymes is still in its infancy. However, a number of laboratories experiment with methods which are adopted from the experience gained with the use of antisense oligodeoxynucleotides for inhibition of gene expression (Uhlmann and Peymann, 1990). These molecules, when applied to a cell culture, apparently are internalized by cells predominantly by pinocytosis, a process which is time and concentration dependent (Stein *et al.*, 1993). The uptake of antisense oligodeoxynucleotides by this approach is poor so that ways of improving it are needed (for a review see Leonetti and Leserman, 1993). Injection is one alternative but this is of no practical value for eventual application. Interaction with the cell membrane can be enhanced by the attachment of large lipophilic groups, e.g., cholesterol, thus improving cellular uptake (Mackellar *et al.*, 1992; Krieg *et al.*, 1993). In addition to attachment of lipophilic groups, the oligonucleotides can be adsorbed to cationic liposomes, such as DOTAP, DOTMA and lipofectamin, for efficient delivery. However, care must be taken to assure that the observed effect is not due to the liposomes alone (Milligan *et al.*, 1993; Stein and Cheng, 1993). More elegant is the entrapment of oligonucleotides in targeted liposomes such as immunoliposomes directed to molecules which are expressed by the targeted cells (Leonetti and Leserman, 1993). This type of delivery seems to be the most efficient of all, as uptake has been reported to be enhanced hundredfold.

In the process of exogenous delivery the ribozyme is exposed to the serum-containing culture medium, which is known to contain significant amounts of RNases (Eder *et al.*, 1991; Emlen and Mannik, 1978; Taylor *et al.*, 1992). To combat degradation by these enzymes, several modifications have been made to the hammerhead ribozyme. RNases utilize the ribose 2′-hydroxyl group to cleave the phosphodiester bond. This degradation can therefore be inhibited either by removal of this group by the incorporation of 2′-deoxyribonucleotides (Taylor *et al.*, 1992; Yang *et al.*, 1992), by a combination of 2′-deoxynucleotides and phosphorothioates (Shimayama *et al.*, 1993) or by the introduction of novel functional groups, e.g., 2′-alkoxy (Goodchild, 1992; Paolella *et al.*, 1992), 2′-fluoro, and/or 2′-amino (Heidenreich and Eckstein, 1992; Pieken *et al.*, 1991). The choice of these substitutions has to be made carefully so as not to interfere with catalytic activity of the ribozyme. It is often found with ribozymes with deoxynucleotide-containing hybridizing arms that the catalytic rate is increased (Shimayama *et al.*, 1993). However, catalytic efficiency as expressed by k_{cat}/K_m is, in most cases, decreased. Interestingly, modifications at the pyrimidine nucleotides are sufficient to enhance stability, indicating that the degrading enzymes are of the RNaseA type. A combination of three phosphorothioates at the 3′-end and the utilization of 2′-fluorocytidine and -uridine throughout the enzyme increased the stability of the ribozyme in serum by approximately three orders of magnitude without unduly compromising the catalytic efficiency (Heidenreich *et*

al., 1994). The presence of the terminal phosphorothioates influences the stabilization to different degrees, depending on the structure of the ribozyme. The more exposed the 3′ end is, the more susceptible it is to degradation by 3′-exonucleases other than RNases. Degradation from the 3′-end can also be inhibited by introducing an unnatural, inverted internucleotidic linkage such as a 5′–5′ or 3′–3′ phosphate diester (Ortigao *et al.*, 1992). Stability is also conferred on the ribozyme by the adsorption on or the entrapment in liposomes as discussed above, by shielding the ribozyme from the RNase-containing culture medium. Thus this mode of delivery has several advantages. It has been shown that hexaethylene glycol linkers can be attached terminally or can replace loop II of the ribozyme (Benseler *et al.*, 1993; Thomson *et al.*, 1993; Marschall and Eckstein, unpublished). Whether this group will facilitate uptake or add to stabilization will have to be determined.

An alternative, although not widely used, to synthesizing modified ribozymes chemically is the preparation of *in vitro* transcripts using T7 RNA polymerase and the required modified nucleoside triphosphates as substrates (Pieken *et al.*, 1991; Aurup *et al.*, 1992). This, however, does not permit the introduction of modifications at selected positions and is obviously limited by the substrate properties of the modified nucleotides. In summary, it has to be admitted that exogenous delivery of ribozymes will have to be the subject of intense study before it can compete with endogenous delivery.

Transport and Localization of the Ribozyme in the Cell

Particularly exogenous delivery of the ribozyme is faced with the proper localization of the ribozyme in the cell to exert its function. Even when the ribozyme has overcome the hydrophobic barriers of the cell membrane to reach the cell's interior, it still has to find its target RNA because the internal space of the cell is subdivided by various membranes into separate compartments. Thus, the ribozyme could be delegated to specific compartments, which are not necessarily those in which the target RNA is to be found. Again, much of this information is inferred from results obtained with antisense oligonucleotides and it has yet to be convincingly demonstrated that ribozymes follow the same pathway. Injection apparently channels the oligomers into the nucleus quite readily (Fisher *et al.*, 1993; Leonetti *et al.*, 1991) Using any of the other techniques for exogenous delivery, the ribozyme will presumably find itself first in the endosome. There it could be trapped and not be available for interaction with RNA. Transfer to the lysosome would be detrimental, as the ribozyme will presumably be subject to degradation there. Receptor-mediated endocytosis, using virus particles containing endosomolytic activity, has been developed for the transfer of DNA, but as yet has to be applied to ribozymes. It represents an improvement in delivery since it helps to release the imported nucleic acid from the endosome into the cytoplasm, bypassing the lysosome (Cotten *et al.*, 1992; Wagner *et al.*, 1992).

The consideration of intracellular transport mechanisms has been addressed recently, using a system of transfected retroviral packaging cells (Sullenger and

Cech, 1993). This system contained two Moloney murine leukemia virus vectors, one encoding a hammerhead ribozyme targeted against *lac*Z RNA, the other encoding *lac*Z. The ribozyme had been assumed to colocalise with the *lac*Z genomic RNA during the packaging process inside the cell. Analysis of the viral progeny and β-gal activity in the host cells demonstrated a drastic reduction of β-gal virus titer. The β-gal protein level remained unaffected, indicating that the viral hammerhead RNA was not able to access and cleave the *lac*Z mRNA in the cytoplasm. However, the colocalization, presumably during packaging, of the ribozyme RNA with the *lac*Z viral RNA led to cleavage. These findings underline the importance of intracellular transport mechanisms for the success or failure of ribozyme applications in cells.

Intracellular stability of ribozymes is not well documented. However, since ribozymes delivered by endogenous transcription or via liposomes are reasonably successful, their lifetime must be sufficiently long to exert their action.

RIBOZYME–SUBSTRATE INTERACTIONS

The hammerhead ribozyme contains two regions which are complementary to the target sequence, 5′ and 3′ to the cleavage site, respectively. They recognize and bind to the substrate to form helices I and III in the ribozyme–substrate complex (Fig. 1). Binding of the substrate occurs in the same manner as it does with antisense RNA so that any inhibitory effect seen with a ribozyme contains a certain contribution resulting from this antisense character. The size of this contribution can be determined using a control ribozyme which has been inactivated by exchanging one or more of the invariant nucleotides in the central core.

The kinetics of cleavage of RNA substrates by the hammerhead ribozyme has been studied in detail (Hertel *et al.*, 1994). Values for the catalytic efficiency, k_{cat}/K_m, of $3 \times 10^5\ M^{-1}\,sec^{-1}$ were found using a short substrate, 17 nucleotides in length, with 16-base pair recognition under single turnover conditions, where for such short substrates the chemical cleavage step is rate limiting. For multiple turnover reactions where product release is rate limiting for this ribozyme, this value is reduced by a factor of approximately 100. The RNA substrate encountered by the ribozyme under *in vivo* conditions will usually be considerably longer, of the order of at least several hundreds of nucleotides in length. Such RNAs will have secondary structures which might interfere with the accessibility of the target sequence. Indeed, the efficiency of cleavage of such long substrates is considerably lower. Thus, a long substrate target, a transcript of the LTR RNA of HIV-1 985 nucleotides in length, displayed an approximately four orders of magnitude lower k_{cat}/K_m, $22\ M^{-1}\,sec^{-1}$, even though it contained a 14-base pair recognition sequence (Heidenreich *et al.*, 1994; Marschall and Eckstein, unpublished). Detailed kinetic analyses of these cleavage reactions have not been undertaken yet so that the reason for the slower cleavage is not precisely known. However, it is generally assumed that binding of the ribozyme to the structured RNA target might contribute to the rate-limiting step (Bertrand *et al.*,

1994). However, it is not certain that such *in vitro* transcripts are good controls for the *in vivo* situation. Proteins could shield the target or they could facilitate the docking of the ribozyme to the target. Indeed, *in vitro* experiments indicate that proteins, such as single-strand binding proteins, actually enhance the catalytic activity by facilitating binding of the ribozyme to the target as well as product release (Tsuchihashi *et al.*, 1993). Turnover under *in vivo* conditions, however, will probably be limited. This poses the question whether turnover is really required or whether stochiometric cleavage is sufficient to inhibit gene expression. This, of course, will depend on the relative amounts of ribozyme and target present in the cell. As mentioned above, high endogenous expression or high exogenous delivery of the ribozyme would obviously be of advantage (Bertrand *et al.*, 1994).

A further complication for the catalytic cleavage of the target might be the presence of sufficient concentrations of Mg^{2+}. It is generally assumed that the Mg^{2+} concentration in the cell is 30 m*M*. However, it is not clear how much of this is present as free Mg^{2+} and not bound to nucleotides or other components in the cell. As the ribozyme requires 10 m*M* Mg^{2+} for maximal activity when assayed *in vitro*, this concentration of free Mg^{2+} should ideally be available at the location where the ribozyme is supposed to be active.

APPLICATIONS

Considerable effort has been put into the development of ribozyme-based inhibition of replication of HIV1. A comparison of results from the various approaches used will hopefully allow the selection of the most promising for further study and application. In addition, much effort has been put into the inhibition of replication of this virus by the antisense strategy with antisense oligodeoxynucleotides. Thus, this system will probably become the most suitable for a comparison of the efficiencies of these two approaches. Because of the importance of the HIV target, it will be dealt with separately but other targets are discussed later.

Ribozymes Directed Against HIV

An early demonstration of hammerhead ribozyme action utilizing endogenous transcription for delivery has been reported for the inhibition of HIV1 replication (Sarver *et al.*, 1990). A plasmid was constructed that carried a ribozyme gene directed against the gag gene of HIV1, placed behind a human-β actin promoter. Using liposomes as transfection agent, an anti-HIV1 gag ribozyme-expressing HeLa $CD4^+$ cell line was established which was clearly less susceptible to HIV1 propagation than ribozyme-minus cells.

As mentioned previously, the type of promoter used will influence the efficiency of ribozyme production. Weerasinghe *et al.* (1991) tested several eukaryotic promoters to drive constitutive and inducible expression of a ribozyme targeted against the HIV1 5' leader region in human T lymphocytes (MT4 cells).

The constitutive promoters of herpes simplex virus thymidine kinase, cytomegalovirus, and SV40 transcribed ribozymes worked well enough for a moderate decrease in the rate of HIV replication and a delay in the production of virus particles of up to 14 days. Resistance to HIV1 propagation during the first 22 days after infection of the cells was achieved with MT4 cells stably transfected with a vector containing a combination of the thymidine kinase promoter and the HIV1 transactivation responsive element (TAR) for driving the ribozyme expression. However, this effect might be due, in part, to a competition for the tat protein, between the vector tk/TAR-promoter and the transactivation of HIV replication.

The HIV1 env gene has also been selected as target for ribozyme cleavage (Chen *et al.*, 1992). A plasmid was constructed containing the genes for up to nine ribozymes in a stretch of up to 400 nucleotides, targeted at different sites in the mRNA for the gp120 part of the env gene. *In vitro* transcripts were more efficiently cleaved by the multiple ribozyme construct than by the monoribozyme. Cotransfection of proviral HIV1 DNA and the plasmid which carries the ribozyme gene driven by either the SV40-, the HIV LTR-, or the β-actin gene promoter into HeLa T4 cells resulted in a significant inhibition of HIV replication as measured by p24 antigen release, paralleled by a drastic reduction of syncytia formation. In agreement with the efficiency of cleavage of the transcript, no inhibition of p24 production was seen with the monoribozyme but a reduction to 10% with the nonaribozyme construct. The intracellular ribozyme action could be demonstrated by isolating and visualising the cleavage products by northern blot analysis. Bertrand *et al.* (1994), however, report that a pentaribozyme was less active than the mixture of the monoribozymes. This difference might again be a reflection of the choice of target sequences.

Homann *et al.* (1993) constructed a plasmid in which a ribozyme directed against the HIV1 gag region was embedded in a long antisense RNA sequence of approximately 400 nucleotides. The activity of this so-called catalytic antisense RNA was compared with that of a catalytically inactive mutant which lacked nucleotide 2.1 in stem I (Fig. 1), next to the invariant region. When SW480 cells were cotransfected with proviral HIV1-DNA and the plasmid with the anti-gag ribozyme, both the active hammerhead and the inactive variant reduced the level of HIV1 replication but the catalytic ribozyme effect was four- to sevenfold stronger than that of the catalytically inactive variant. The inhibitory activity of the inactive mutant should represent the contribution of the long antisense flanks of this particular construct.

Another successful application of the catalytic properties of ribozymes for the inhibition of HIV1 replication has been reported with a hairpin ribozyme targeted against the HIV1 leader sequence (Ojwang *et al.*, 1992; Yu *et al.*, 1993). Cotransfection of a plasmid containing the HIV proviral DNA with a plasmid or a retroviral vector carrying the hairpin ribozyme gene attached to the human β-actin promoter (or human tRNA promoter or adenovirus *VA1* promoter) into HeLa cells resulted in a reduction of HIV1 replication of up to 80% in a transient assay. The efficiency of inhibition depended on the HIV plasmid/ribozyme plasmid ratio and on the promoter chosen. The efficiency of inhibition again depended on the type of promoter used and also on its position relative to the

gene for the ribozyme. A mutant ribozyme, inactivated by exchange of the three consecutive invariant nucleotides A12–A14 in loop 2 for UGC, led to only 10% inhibition of HIV replication, which indicates that the anti-HIV effect is cleavage dependent. Further studies have shown that this hairpin ribozyme cleaves not just one particular, but diverse HIV1 strains, making it a potential tool for gene therapy treatment of HIV1-infected patients.

These studies have been extended to stable cell lines which constitutively express the ribozyme gene (Yamada *et al.*, 1994). No virus infectivity was detectable in cell cultures expressing the ribozyme up to 35 days after viral challenge. Interestingly, the ribozyme apparently not only cleaved and thus inactivated viral mRNA but also interfered with early events of infection by cleaving the viral RNA. These are very encouraging results for intracellular immunisation of human T cells against HIV1 replication with ribozymes.

Haseltine and co-workers found that HIV1 replication in human T cells is better inhibited by an antisense RNA complementary to the HIV1 tat region than by a ribozyme targeted against the same site (Lo *et al.*, 1992). Both the antisense RNA- and the ribozyme-expressing Jurcat cells produced significantly less HIV-1 progeny than the parental cells when challenged with HIV-1, but the antisense RNA-producing cells additionally retarded the breakthrough in HIV production by 4 days.

Crisell *et al.* (1993) found that ribozymes which cleave a transcript *in vitro* very poorly are very efficient for the inhibition of HIV1 replication in human T cells infected with HIV particles. The ribozyme with arms each 33 nucleotides long showed the strongest inhibitory effect, beyond which the anti-HIV1 effect decreased. A mutant ribozyme, inactivated by replacement of guanosine at position 5, with flanking arms 45 and 70 nucleotides in length, respectively, was ineffective.

Successful application of ribozyme cleavage of a HIV-1 RNA has also been accomplished in a prokaryotic system (Sioud and Drlica, 1991). A hammerhead ribozyme targeted against the HIV1 integrase mRNA, when transcribed from a plasmid in an integrase-producing *E. coli* strain, abolished the production of integrase mRNA and protein. This was not observed with an antisense control, which binds to the same target site as the ribozyme but lacks its core domain. In addition to that, the antiintegrase ribozyme showed the same inhibitory effect in an RNaseIII-deficient *E. coli* strain, which supports the interpretation that the inhibition is due to ribozyme cleavage and not an antisense effect, since RNase III is thought to be responsible for antisense inhibition in prokaryotes (Krinke and Wulff, 1990).

Cancer and Other Targets

Targets other than HIV have also been investigated. Some examples are given in this section. The fusion gene BCR/ABL is the cause of chronic myelogenous leukemia (CML). This gene codes for two mRNA types, B3A2 and B2A2, which are both translated into a tyrosine kinase of 210kDa, unique to the malignant cell phenotype. Short synthetic ribozymes, directed at this RNA, were

delivered with the lipofection agent DOTAP to the CML cell line K562 which carries this gene. The expression of this protein was reduced after 72 hr to 23% with the active ribozyme and to 43% with a mutant which had been inactivated by exchanging guanosine at position 10 for an adenosine (Lange *et al.*, 1994). In similar experiments with a synthetic DNA–RNA hybrid ribozyme complete inhibition of expression of this protein was observed. It was somewhat more efficient than a control antisense oligodeoxynucleotide (Snyder *et al.*, 1993). The mechanism by which antisense oligodeoxynucleotides exert their function probably involves the activity of RNaseH to destroy the RNA once the oligodeoxynucleotide has annealed to the target RNA. Thus, it might not be surprising that this antisense oligodeoxynucleotide was almost as inhibitory as the ribozyme. These results are encouraging for *in vivo* experiments and may ultimately lead to treatment of patients with CML.

Another important target for ribozyme treatment are genes involved in tumorigenicity and cancer (Kashani *et al.*, 1992). Ribozymes have been shown to alter the morphology and growth behavior of human bladder carcinoma cells (EJ) in tissue culture and their malignant potential in mice after transurethral implantation when endogenously transcribed from an expression vector, resulting in an almost twofold increased survival rate of ribozyme-treated mice. Suppression of transformation of cells, to a certain extent, has also been achieved by intracellular hammerhead-mediated cleavage of an oncogene mutant of the c-Ha-ras gene (Koizumi *et al.*, 1992). When NIH3T3 cells, continuously expressing an anti-c-Ha-Ras ribozyme, were transfected with a plasmid carrying the activated c-Ha-Ras gene behind a RSV promoter, a disappearance of the c-Ha-Ras mRNA and an approximate 50% reduction of focus formation was observed compared to transformed cells which harbored only a control plasmid with no ribozyme gene. To prove the cleavage specificity of the observed effects, the sequence of the substrate RNA and not of the ribozyme was altered to prevent cleavage. A control experiment with an uncleavable but activated c-Ha-Ras mRNA led to no suppression of transformation.

An exogenously applied hammerhead ribozyme resulted in cleavage of TNFα mRNA in human promyelocytic leukaemia cells (HL60 cell line) (Sioud *et al.*, 1992). This ribozyme contained at its 3′ end the T7 transcription terminator to stabilize it against degradation by nucleases from the 3′ end. Transfection of the cells with the *in vitro* transcribed active hammerhead ribozyme reduced the TNFα mRNA to 10% and that of the protein to 150 fmol/ml. As control a RNA was used with the same sequences as the hammerhead ribozyme except that the core region was deleted. It reduced the TNFα mRNA level only to 60%; that of the protein to 400 fmol/ml.

Bovine leukemia virus (BLV) encoded at least two regulatory proteins, Rex and Tax. The gene for a hammerhead ribozyme which cleaves transcribed portions of the *rex/tax* mRNa *in vitro* was cloned into an expression vector and transfected into BLV-infected lung cells (Cantor *et al.*, 1993). Intracellular cleavage of the mRNA was confirmed by reverse transcriptase PCR. Expression of the core protein p24 was inhibited 61%; that of reverse transcriptase, 92%.

These results suggest that cattle expressing these ribozyme sequences may be able to control BLV replication.

Expression of c-*fos* gene has also been intervened with to study the role of the Fos gene product in drug resistance and in mediating DNA synthesis and repair processes (Scanlon *et al.*, 1991). The ribozyme effects were compared with control experiments using inactive ribozyme variants. The active ribozyme resulted in a sevenfold stronger suppression of the c-fos phenotype than the inactive one.

Vector-mediated endogenous expression of a hammerhead ribozyme which is targeted against the α-lactalbumin mRNA in mouse C127 I mammary cells resulted in a 60–80% inhibition of α-lactalbumin mRNA paralleled by significant down-regulation of the intracellular level of the corresponding protein (L'Huillier *et al.*, 1992). The control experiment with an catalytically inactive variant led to no change on the mRNA level and only a small reduction of intracellular α-lactalbumin, indicating that the antisense properties of the ribozyme used in this study contribute only weakly to the overall inhibitory effect.

A recent study utilizes hammerhead ribozymes for the investigation of the biological function of the *Drosophila melanogaster fushi tarazu* (ftz) gene (Zhao and Pick, 1993). Transgenic flies were generated carrying an anti-ftz hammerhead ribozyme behind the heat-inducible hsp70 promoter. Heat-shock treatment of *Drosophila* larvae at different developmental phases caused different disruptions in the phenotype pattern monitored by staining methods. Strong disruption phenotypes were never seen when a mutant ribozyme, inactivated by replacement of nucleotides 6, 9, and 13 (Fig. 1), or an antisense RNA lacking the ribozyme catalytic core was used instead of the active hammerhead. Here ribozymes provide a novel means for the elucidation of gene function under the complex requirements of a developing multicellular organism.

A plasmid containing the gene for a ribozyme directed against the *white* gene responsible for eye pigmentation in *Drosophila melanogaster* has been used to establish a transgenic line carrying homocygous copies of this construct (Heinrich *et al.*, 1993). A reduction of pigmentation could be observed in a genetic background with *a priori* low levels of *white* gene expression.

A comparative analysis of ribozyme, antisense RNA, and antisense DNA and antisense oligodeoxynucleotides for inhibition of the processing of small ribonucleoprotein U7-dependent histone pre-mRNA in nuclear extracts has been reported (Cotten *et al.*, 1989). This *in vitro* processing was inhibited by a sixfold excess of antisense RNA over the U7 sequence, a 60-fold excess of DNA complementary to the entire U7 sequence, a 600-fold excess of an 18-mer antisense oligodeoxynucleotide, and a 1000-fold excess of ribozyme. Thus, at least in this system the ribozyme would appear to be the least efficient. Ribozyme-mediated cleavage of the U7 snRNA was repeated by injection of ribozyme-coding sequences placed between the promoter sequences for a *Xenopus* tRNA and injected into the oocytes (Cotten and Birnstiel, 1989). The ribozyme RNA remained localized mainly in the nucleus, whereas the substrate RNA migrated rapidly into the cytoplasm. However, sufficient ribozyme was present in the

cytoplasm to destroy the substrate RNA. Again, it was concluded that the ribozyme was quite inefficient in cleavage as a 500- to 1000-fold excess of ribozyme over substrate was necessary for complete cleavage.

Antisense vs Catalytic Effect

Any ribozyme construct contains two regions which are complementary to the substrate sequence and which are required for substrate binding. This interaction might evoke an antisense RNA effect which, in its simplest form, will interfere with translation. Unfortunately, the mechanisms of inhibition of gene expression by antisense RNA are not well understood so that the inhibitions observed might have more complicated causes (Nellen and Lichtenstein, 1993). However, to evaluate the contribution of catalysis to the inhibition observed with a ribozyme, the potential antisense effect should be determined. This can be done in two ways. Either at least one of the invariant nucleotides should be exchanged to destroy the catalytic power of the ribozyme or a molecule prepared without the core region of the ribozyme. Any inhibitory activity seen with the ribozyme in excess over either of these controls can be attributed to the catalytic activity of the ribozyme. To support this, it is desirable to demonstrate that the target RNA has indeed been cleaved. However, complications can arise when nucleases such as RNaseIII might be present which can cleave double-stranded regions of RNA. In the examples discussed in this review, these controls are mentioned wherever they have been applied. In general, the active ribozyme is more efficient than the controls, although the degree of difference can vary considerably. At present there are not sufficient experimental data to allow a generalization of the size of the catalytic effect. The magnitude of this effect might also depend on the system under study.

This antisense RNA effect should be distinguished from the inhibitory effect seen with antisense oligodeoxynucleotides (Uhlmann and Peymann, 1990; Milligan *et al.*, 1993; Ratajczak and Gewirtz, 1994). The inhibitory effect of these antisense oligodeoxynucleotides is due, at least in part, to the activity of RNaseH, which cleaves the RNA component of DNA/RNA hybrids. Thus, although these oligonucleotides are not inherently catalytically active, they induce such an activity. This activity is not associated with the ribozyme. However, it could become important when chimeric ribozymes, containing deoxynucleotides, are used (Shimayama *et al.*, 1993; Taylor *et al.*, 1992; Yang *et al.*, 1992). It is therefore not *a priori* certain that ribozymes are superior to the action of such oligodeoxynucleotides. Whether one or the other is the method of choice might, again, depend on the individual case.

OUTLOOK

The results discussed here demonstrate the successful use of ribozymes, in particular the hammerhead, for the inhibition of gene expression in cell culture, i.e., *ex vivo*. Clearly, the extension of this work to *in vivo* systems is highly desirable and it is expected that the first results of such efforts will be available

soon. Endogenous delivery of the ribozymes is favored at present over exogenous application. This is probably a reflection of the difficulties encountered in obtaining sufficient amounts of modified, RNase-resistant ribozymes by chemical synthesis. However, this will presumably change as chemical synthesis is improved, as happened with antisense oligodeoxynucleotides. For both approaches, delivery is a major problem. Thus selection of a suitable vector for the endogenous delivery and choice of liposome or some other modification to facilitate uptake of the ribozyme for the exogenous delivery mode are crucial for the success of this method. A more rational approach to select the best target sequence is also desirable. The ribozyme-based inhibition of gene expression will also have to be compared with alternative methods such as the antisense oligonucleotide- and the antisense RNA-based methods. At this early stage it is difficult to say whether one will really be more efficient than the other or more suitable for application in general or whether this will depend on the target. In any case, the advent of ribozymes has considerably increased our armoury for interference with gene expression.

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